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# Protection by Catatoxic Steroids against Cyclophosphamide-Induced Organ Lesions

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*Summary.* In rats, fatal cyclophosphamide intoxication can be prevented by certain catatoxic steroids, particularly PN (pregnenolone-16α-nitrile), SC-11927 and spironolactone. Numerous other steroids, known to possess catatoxic activity against different poisons fail to prevent cyclophosphamide intoxication.

Most catatoxic effects are due to the induction of hepatic microsomal drug-metabolizing enzymes but phenobarbital, the prototype of nonsteroidal catatoxic compounds, does not protect against cyclophosphamide.

The literature on catatoxic steroids is briefly surveyed especially emphasizing its bearing upon the pluricausal concept of disease. Many examples are cited to show that often in pathology, the apparently direct and specific effects of pathogens or curative drugs are actually indirect and nonspecific in that they are mediated through a common relay station (e.g., hepatic enzyme induction) which is essential for the maintenance of homeostasis in many, essentially different, circumstances.

*Zusammenfassung.* Verschiedene katatoxische Steroide, darunter besonders PN (Pregnenolon-16α-nitril), SC-11927 und Spironolacton, verhüten bei der Ratte eine letale Cyclophosphamidvergiftung. Andere Steroide, die sich gegen verschiedene Gifte katatoxisch bewährt haben, blieben gegen die Cyclophosphamidvergiftung wirkungslos.

Die meisten katatoxischen Wirkungen beruhen auf der Induktion mikrosomaler Leberenzyme, die den Abbau von Giften beschleunigen; trotzdem gewährt Phenobarbital, ein Prototyp der nichtsteroiden Enzyminduktoren, keinen Schutz gegen Cyclophosphamidvergiftung.

Anhand einer Übersicht der Literatur über katatoxische Steroide wird besonders ihre Rolle bei den sogenannten plurikausalen Krankheitserscheinungen betont. An vielen Beispielen wird gezeigt, daß häufig anscheinend direkte und spezifische Effekte von Pathogenen oder Heilmitteln tatsächlich indirekt und unspezifisch sind. Derartige Wirkungen werden oft auf dem Umwege über ein gemeinsames Zwischenglied in einer Reaktionskette ausgeübt (z.B. durch eine Einwirkung auf die Induktion von Leberenzymen), das für die Aufrechterhaltung der Homöostase in vielen grundsätzlich verschiedenen Situationen notwendig ist.

The therapeutic and toxic actions of cyclophosphamide are very similar to those of other nitrogen mustards. Its absorption, metabolism and excretion have been studied in some detail (Calabresi and Welch, 1965), but comparatively little attention has been given to its experimental pathology and the possible protective effect of steroids.

It is well-known that cyclophosphamide as such is relatively inert and requires activation *in vivo*, probably in the blood, liver or both (Calabresi and Welch, 1965). The compound also has no cytotoxic effect upon Yoshida sarcoma cells *in vitro* but is rapidly transformed into an active compound *in vivo*. The latter is demonstrable in the blood and urine by bioassay if added to tumor cells *in vitro*. In completely hepatectomized rats, only a small fraction of cyclophosphamide is thus activated. Since pretreatment of rats with the micro-

somal enzyme inhibitor SKF 525-A inhibits *in vivo* activation, the latter process presumably takes place in hepatic microsomes (Brock and Hohorst, 1962).

In mice SKF 525-A, and the related microsomal enzyme inhibitor Lilly 18947, reduced the lethality of cyclophosphamide while pretreatment with the microsomal enzyme inducer, phenobarbital, did not change it. Curiously "neither SKF 525-A" nor phenobarbital had an effect on the antitumor efficacy of cyclophosphamide. Mice housed on cedar chip bedding (also known to be a microsomal enzyme blocker) were less susceptible to the lethal effects of cyclophosphamide, but tumor bearing mice on this bedding showed greater antitumor response to the drug than those on hardwood bedding (Hart and Adamson, 1969).

Virtually nothing is known about the effect of steroids upon cyclophosphamide intoxication. Allegedly, in rabbits, the toxic effect of this compound upon hemopoietic organs and the adrenals is aggravated by pretreatment with prednisolone (Fleischer and Riedel, 1964). Addition of prednisolone to liver slices inhibits cyclophosphamide activation *in vitro*. Hence, it was assumed that prednisolone hydroxylation and cyclophosphamide activation compete for the $NADPH_2$ dependent drug-metabolizing enzyme system (Hayakawa *et al.*, 1969). The anabolic steroid $\Delta$ '-17$\alpha$-methyltestosterone failed to prevent the cytostatic effect of cyclophosphamide upon Jensen sarcomas in rats (Lutzmann and Schmidt, 1965).

Earlier observations showed that an extraordinary degree of resistance to numerous toxicants can be induced by certain steroids (Selye, 1970a), hence we undertook systematic investigations on this type of protection against cyclophosphamide.

It has long been known that hormones can greatly alter the resistance of the body to various types of injuries. Our own interest in this matter stems from the observation that increased glucocorticoid production is a characteristic feature of the alarm reaction, elicited by any stressor. This endocrine response helps to maintain homeostasis since resistance to stress is greatly impaired after adrenalectomy but can be restored to near normal levels by exogenous corticoids (Selye, 1936, 1950).

Curiously, stress produced by many agents can raise tolerance to a variety of unrelated toxicants. Often this stress-induced nonspecific or "cross-resistance" results from an increased corticoid secretion (Selye, 1961). However, our hopes of raising the stress tolerance of intact animals far above the norm by treatment with glucocorticoids did not materialize, presumably because a near optimal corticoid supply is assured by the physiologic activity of the adrenal cortex (Selye, 1950). Actually in intact animals, glucocorticoids offer considerable protection only against a few agents, such as inflammatory irritants (Selye, 1950), bacterial endotoxins (Selye, 1966a), lathyrogens (Selye, 1957) and certain ganglioplegics (Selye, 1970k). It seems that glucocorticoids act as "syntoxic" hormones, by merely suppressing pathologic tissue reactions; thus they maintain a balanced "symbiosis", or co-existence, with pathogens (e.g., inflammatory irritants) without destroying the latter.

Considerable progress has been made in the elucidation of steroid induced resistance recently when it became clear that there exists a special class of "catatoxic steroids", which —unlike the syntoxic glucocorticoids—do not merely suppress the body's response to pathogens but actually attack the toxicants. Usually, though not always, this is achieved by increasing the rate of metabolic degradation in the liver. The catatoxic action is independent of any other known hormonal effect, although it is often associated with anabolic (ethylestrenol, norbolethone, oxandrolone) or antimineralocorticoid (spironolactone, SC-11927, spiroxasone) properties. Among the chemical characteristics which appear to enhance the catatoxic potency of steroids are the possession of nitrile or lactone groups and a 19-nor configuration (Selye, 1970b).

As stated in the introduction, the toxicity of cyclophosphamide is increased during its metabolism *in vivo*. It seemed possible, however, that by further enhancing the biotransformation of the resulting toxic product the latter might be degraded to metabolites which are again inactive. To explore this possibility we purposely selected a number of steroids with catatoxic, syntoxic or no known detoxifying properties, as well as a few control substances such as thyroxine and phenobarbital which likewise influence microsomal drug-metabolizing enzyme induction.

## Materials and Methods

Female ARS/Sprague-Dawley rats with a mean initial body weight of 100 g (range 90–110 g) were maintained exclusively on Purina Lab Chow and tap water, divided into equal groups of 10 rats each and treated as outlined in the Table. To obtain the best catatoxic effect, it is important to allow a few days of pretreatment; hence all animals received *cyclophosphamide* (Horner) at the dose of 10 mg in 0.2 ml water s.c. daily, beginning only on the fourth day of treatment with the potentially catatoxic substances.

The following steroids were tested for possible protective or sensitizing effects:

*PN* [3$\beta$-hydroxy-20-oxo-5-pregnene-16$\alpha$-nitrile (Searle)], *spironolactone* (Searle), *SC-11927* [potassium 3-(3-oxo-9$\alpha$-fluoro-11$\beta$, 17$\beta$-dihydroxy-4-androstene-17$\alpha$-yl) (Searle)], *norbolethone* (Wyeth), *ethylestrenol* (Organon), *progesterone* (Schering), *oxandrolone* (Searle), *prednisolone acetate* (Roussel), *triamcinolone* (Lederle), *desoxycorticosterone acetate*, *DOC-Ac* (Schering), *hydroxydione sodium hemisuccinate* (Pfizer) and *estradiol* (Roussel). For comparative purposes we also tested a nonsteroidal microsomal enzyme inducer, *phenobarbital* (BDH) and *thyroxine* (BDH) which in many instances had previously been shown to interfere with this type of enzyme induction.

The first experiment (Table) was terminated on the 15th, the second (not tabulated) on the 12th, day. Throughout the entire period of observation in both experiments, all steroids were administered at the dose of 10 mg, and phenobarbital at the dose of 6 mg in 1 ml water p.o. twice daily; thyroxine was injected s.c. (in the form of its Na salt) at the dose of 200 $\mu$g in 0.2 ml water once daily.

The Table lists the final body weights (grams) and the mortality rates (%). The significance of the apparent differences between the final body weights of the experimental animals and the controls was determined by Student's *t*-test. The mortality rates were arranged in a $2\times2$ contingency table and their statistical significance computed by the "Exact Probability Test" of Fisher and Yates (Siegel, 1956; Finney, 1948).

For histologic study specimens of various tissues, fixed in picric-acid-Susa solution, were embedded in paraffin and stained with the PAS-technique.

## Results

As shown by the Table, weight gain was completely inhibited and mortality was 100% in the controls treated with cyclophosphamide alone. On the other hand, PN, spironolactone and SC-11927, all steroids previously shown to possess high catatoxic potency against other substrates, also offered virtually perfect protection here, in that they permitted normal growth and completely prevented mortality. Norbolethone and ethylestrenol offered no significant protection against mortality but these strong anabolics did permit significant (though subnormal) body weight gains. Progesterone which is devoid of anabolic potency, nevertheless, offered considerable protection against mortality. As judged by earlier work, oxandrolone is a fairly potent anabolic and catatoxic steroid, yet it was quite devoid of anticyclophosphamide activity with respect to either body weight gain or mortality.

The two glucocorticoids, prednisolone and triamcinolone, as well as estradiol or thyroxine, all of which have catabolic effects, diminished the body weight significantly below the control levels. Since all four of these catabolic compounds also accelerated mortality, the body weights listed in the Table for these groups had to be taken on the 12th day, whereas those of all other rats correspond to the 15th day, when mortality reached 100% in the controls and the experiment was terminated.

The mineralocorticoid, DOC and the strongly anesthetic hydroxydione offered no protection either against the stunting of body growth or against mortality.

Fig. 1. Effect of catatoxic steroids upon cyclophosphamide-induced labial lesions. Both rats received cyclophosphamide. Left: Additional treatment with ethylestrenol fails to prevent the characteristic swelling, hemorrhage and necrosis of the lips. Right: PN offers complete protection

Table. *Protection by catatoxic steroids against cyclophosphamide intoxication*

| Treatment | Final body weight (g) | Mortality (%) |
|---|---|---|
| None | 101 ± 2 | 100 |
| PN | 140 ± 4*** | 0*** |
| Spironolactone | 123 ± 4*** | 0*** |
| SC-11927 | 134 ± 3*** | 0*** |
| Norbolethone | 117 ± 2*** | 90 N.S. |
| Ethylestrenol | 113 ± 2** | 90 N.S. |
| Progesterone | 103 ± 2 N.S. | 30*** |
| Oxandrolone | 99 ± 3 N.S. | 100 N.S. |
| Prednisolone-Ac | 74 ± 1*** | 100 N.S. |
| Triamcinolone | 62 ± 2*** | 100 N.S. |
| DOC-Ac | 95 ± 3 N.S. | 100 N.S. |
| Hydroxydione | 95 ± 2 N.S. | 100 N.S. |
| Estradiol | 80 ± 3*** | 100 N.S. |
| Thyroxine | 91 ± 3* | 100 N.S. |
| Phenobarbital | 107 ± 4 N.S. | 90 N.S. |

*** = $P < 0.005$; ** = $P < 0.01$; * = $P < 0.05$; N.S. = Not Significant. Asterisks indicate significance of protection, underlined asterisks significance of aggravation. The mean survival of each group is not listed, but all rats treated with thyroxine (which developed hemorrhagic pericarditis) and most of those treated with prednisolone, triamcinolone and estradiol (the strongly catabolic steroids) died before the controls

Phenobarbital, the prototype of the hepatic microsomal enzyme inducing drugs, which accelerates the detoxication of many other drugs, was likewise ineffective in protecting the rats against cyclophosphamide.

Most of the untreated controls, as well as the rats treated with compounds devoid of protective potency, exhibited pronounced edema and hemorrhages around the snout (Fig. 1), perhaps as a consequence of infection with normally innocuous

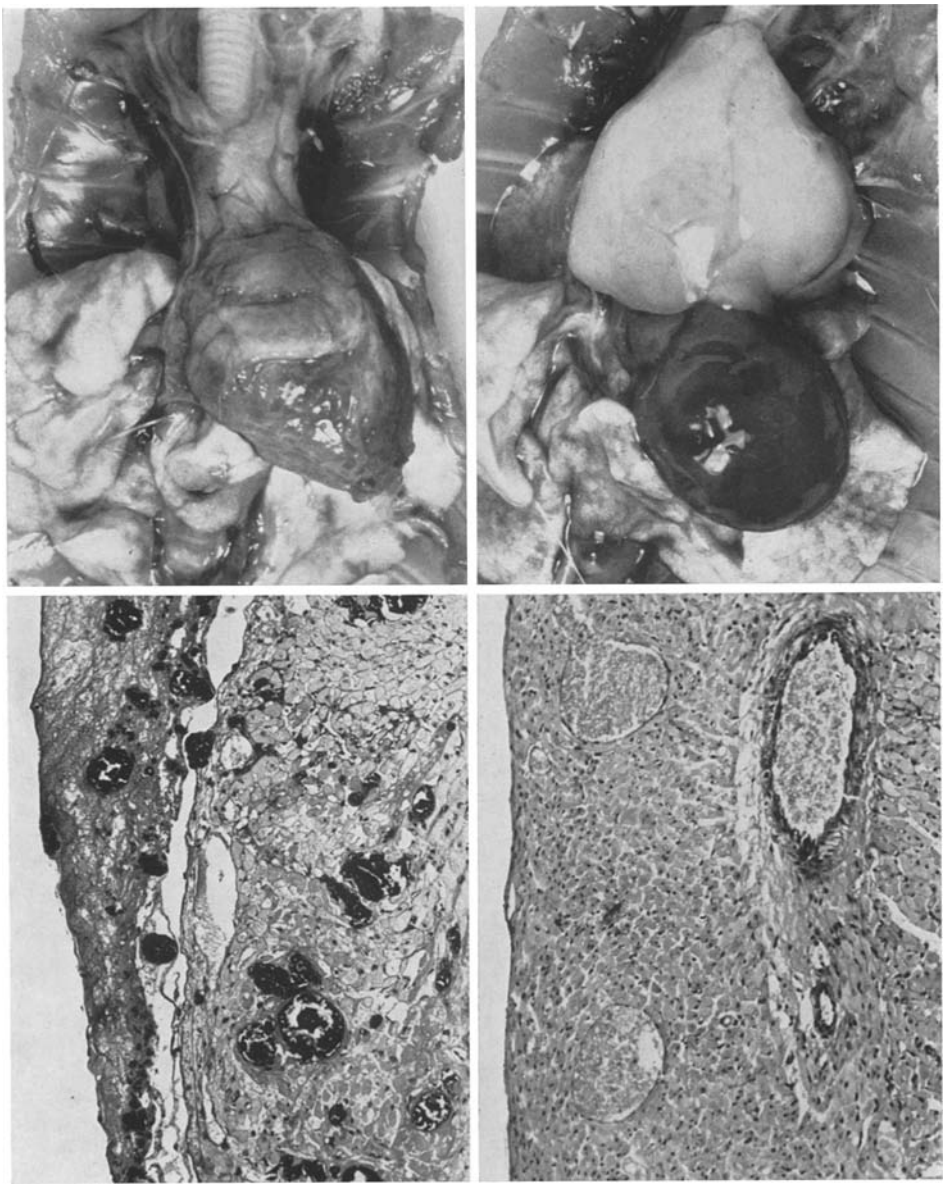

Fig. 2. Effect of catatoxic steroids upon cyclophosphamide-induced cardiac lesions. Both rats received cyclophosphamide. Top left: Ethylestrenol fails to prevent the development of necrotizing myocarditis (light areas near base of the heart) and fibrinous pericarditis. The virtually complete atrophy of the thymus reflects the intense stress reaction which was also associated with pronounced adrenocortical enlargement. The thymic lymph nodes (as those in other parts of the body) are hemorrhagic. Top right: Complete prevention of cardiac lesions by PN. The thymus is normally developed. Bottom left: Numerous bacterial colonies in myocardium and in the fibrinous pericardial covering. Complete absence of leukocytic infiltration. Multiple myocardial necroses. Bottom right: Normal pericardium and myocardium. PAS. $\times 84$

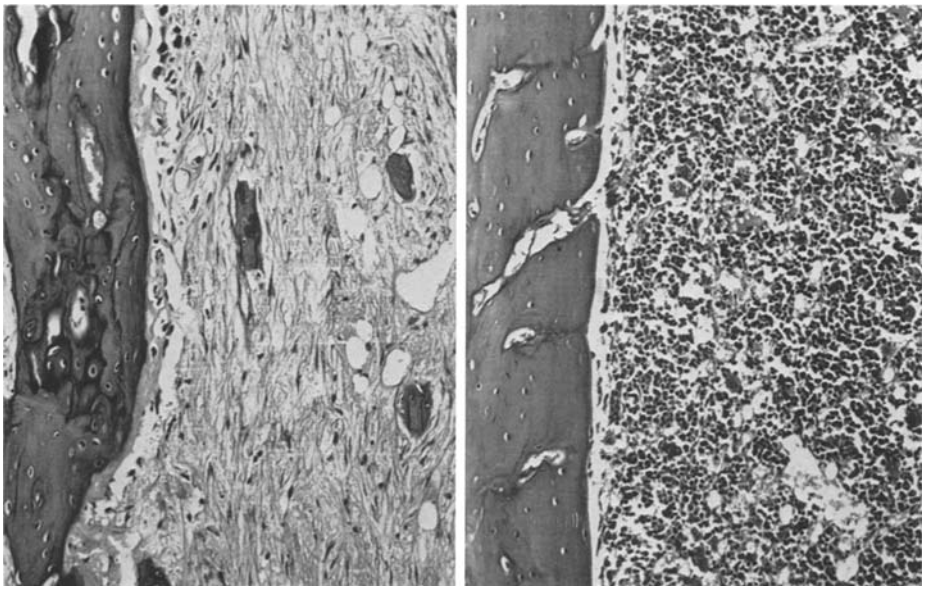

Fig. 3. Protection of bone marrow against cyclophosphamide by SC-11927. Left: In the femur of rats given cyclophosphamide alone the bone marrow consists virtually only of gelatinous connective tissue. Right: Femur of rat which in addition to cyclophosphamide was treated with SC-11927. The bone marrow is essentially of normal appearance. PAS. ×84

microorganisms, since cyclophosphamide notoriously causes leukopenia and depresses immunologic resistance. This increased tendency to infection became even more obvious at autopsy because of the frequent occurrence of pericarditis, myocarditis and, less commonly, renal abscesses in most groups other than those protected by catatoxic steroids. In general, the pericarditis and myocarditis were macroscopically characterized by yellowish areas of fibrin deposits and necroses (Fig. 2), only in the thyroxine pretreated rats was it consistently associated with hemorrhage. These gross observations could be confirmed histologically. Numerous microbial colonies developed in the fibrinous pericardial deposits and within necrotic areas of the cardiac muscle and kidneys, but suppuration was not seen because of the leukopenia. The predominantly cardiac localization of very acute hemorrhagic lesions in the thyroxine treated group may have been due to the stimulation of cardiac work characteristic of this hormone. In this connection it is noteworthy, however, that cyclophosphamide also aggravates Chagas myocarditis in mice inoculated with T. cruzi, presumably through its immunosuppressive effect (Kumar *et al.*, 1968).

In the unprotected animals, hemorrhages were also seen in most of the lymph nodes, which assumed the aspect of "hemolymph nodes"; the lymph vessels, including the thoracic duct, were filled with blood. The tendency to bleed also manifested itself by multiple hemorrhages, especially at subcutaneous injection sites, into the gastrointestinal tract and, especially in the ethylestrenol group, into the uterus. Eventually most of the unprotected animals became extremely anemic.

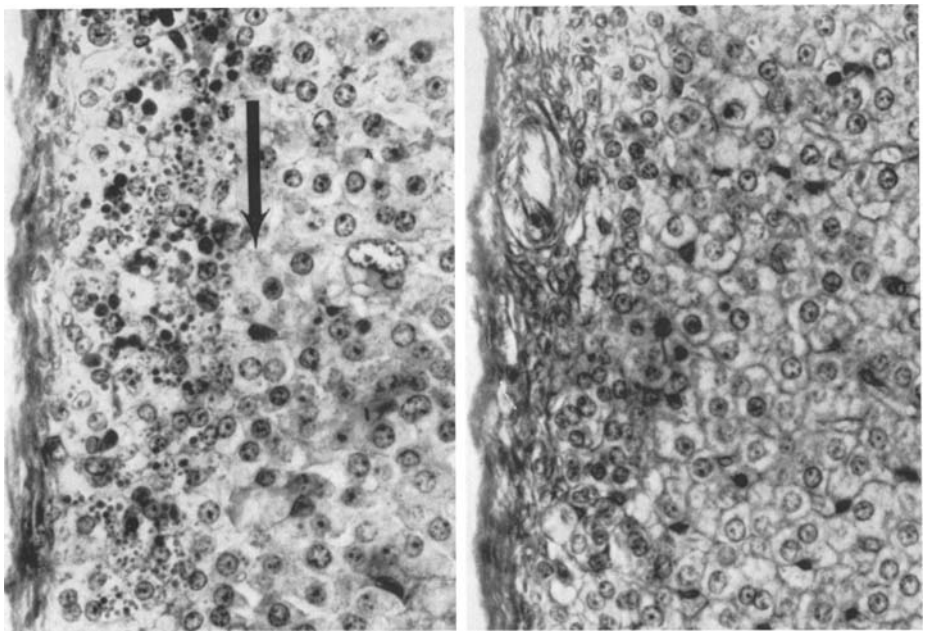

Fig. 4. Effect of PN upon cyclophosphamide-induced adrenocortical changes. Left: In a rat given cyclophosphamide alone, the glomerulosa of the greatly enlarged adrenal (to left of arrow) is laden with PAS-positive granules. Right: Additional treatment with PN prevents this change. PAS. ×235

Histologic examination of the bone marrow revealed that the hemopoietic elements were almost completely replaced by loose, gelatinous connective tissue (Fig. 3). The lymphoid elements virtually disappeared from the lymph nodes and spleen. The glomerulosa of the greatly enlarged adrenal cortex was heavily laden with PAS-positive granules (Fig. 4). All these changes were totally prevented by PN, SC-11927 or spironolactone.

The experiment was repeated under identical conditions except that it was terminated on the 12th day when the mortality in the controls was still only 70%. Here, the activities of the various treatments were almost exactly the same as in the first series, hence the results of this repeat experiment need not be tabulated. It is worthy of mention, however, that the animals again exhibited the curious protection by progesterone against mortality but not against body weight loss, the protection by ethylestrenol and norbolethone against body weight loss but not against mortality, the production of hemorrhagic pericarditis by thyroxine and the aggravation of catabolism and enhancement of mortality by glucocorticoids and estradiol. The total inefficacy of phenobarbital as a protector against cyclophosphamide was also confirmed.

## Discussion

In order to evaluate these experiments, it is essential to review at least the highlights of earlier work on the effect of catatoxic steroids upon other types of intoxications.

The extraordinarily broad activity spectrum of these steroids is illustrated by the following partial list of toxicants against which they offer protection: numerous digitalis alkaloids

(Selye, 1969a; Selye *et al.*, 1969a–c, 1970a), indomethacin (Selye, 1969b), various anesthetics and hypnotics, including many barbiturates and steroids (Selye, 1970c; Selye *et al.*, 1969d, e, 1970a), dimethylbenzanthracene, or DMBA (Kovacs and Somogyi, 1969, 1970), and its highly active metabolite 7-OHM-MBA (Somogyi and Kovacs, 1970), nicotine (Selye *et al.*, 1970b), mephenesin (Selye, 1970d), picrotoxin (Selye, 1970e), phenindione (Selye, 1970f), bishydroxycoumarin (Solymoss *et al.*, 1970c), hypervitaminosis A (Tuchweber and Garg, 1970), cycloheximide (Selye, 1970g), meprobamate (Selye, 1970h), colchicine (Selye, 1970l), methyprylon (Selye, 1970i), and a great variety of pesticides (Selye, in press a). Catatoxic compounds also protect against the diverse forms of infarctoid cardiopathies that are produced by steroids or digitoxin on certain diets (Selye, 1970j). Even the fatal renal damage produced by $HgCl_2$ can be prevented by certain catatoxic steroids but, in this respect, only those (spironolactone, isoxasone, emdabol) containing thioacetyl groups are active (Selye, 1970m; Selye *et al.*, in press). Evidently, the protection offered by these steroids extends to compounds of vastly different chemical structure and pharmacologic activity.

The fact that hepatic detoxication plays a role in this type of drug resistance has first been called to our attention in 1931, when we found that, in rodents, the anesthetic effect of tribromoethanol is greatly prolonged by removal of about 70% of the liver (Waelsch and Selye, 1931). Subsequently, the same operation was shown to increase the duration of steroid anesthesia (Selye, 1941) and to augment the toxicity of indomethacin (Selye, in press b). Furthermore, the protective effect of catatoxic steroids is associated with a proliferation of the smooth endoplasmic reticulum (SER) in hepatocytes (Gardell *et al.*, 1970a; Kovacs *et al.*, 1970), an increase in hepatic weight and mitotic index, as well as an accelerated regeneration of the liver after partial hepatectomy (Horvath *et al.*, 1970).

The understanding of the catatoxic effect was greatly promoted by the realization of a relationship between the observations just mentioned and completely independent investigations performed in many other laboratories, which showed that drug-metabolizing hepatic microsomal enzymes can be induced by various chemicals, including steroids (Axelrod, 1956; Booth and Gillette, 1962; Brodie, 1956; Conney, 1967; Kato *et al.*, 1962). At first the induction of drug-metabolizing enzymes by steroids was variously ascribed to their glucocorticoid, testoid or anabolic potencies. However, it is now clear that a similar mechanism of hepatic enzyme induction is also responsible for many of the protective catatoxic steroid actions irrespective of all their classic hormonal porperties. This has been demonstrated in our Institute by the observation that, parallel with their *in vivo* effects upon resistance, these steroids accelerate the blood clearance of many toxic substrates (Solymoss *et al.*, 1970a, c; Solymoss *et al.*, in press a, b). Furthermore, increased enzyme activity was noted even upon *in vitro* incubation of substrates with the microsomal + supernatant fractions of the liver of animals which were treated *in vivo* with catatoxic steroids (Solymoss *et al.*, 1969, 1970b).

Finally, the protective potency of catatoxic steroids was found to be impaired in animals pretreated with a variety of compounds known to block the activity of the induction of hepatic microsomal enzymes, namely: ethionine (Gardell *et al.*, 1970b), metyrapone (Selye and Mécs, 1970), SKF 525-A (Solymoss *et al.*, in press b, 1970a), cycloheximide, actinomycin D and puromycin (Solymoss *et al.*, 1970 d).

In the present experimental series, the most striking observations were that: three catatoxic steroids (PN, SC-11927 and spironolactone) induce an extraordinary degree of resistance against cyclophosphamide, whereas other compounds (ethylestrenol and norbolethone) known to possess considerable catatoxic potency against many poisons (e.g., dihydrotachysterol, digitoxin, indomethacin) are virtually without influence upon cyclophosphamide intoxication. It is also remarkable that phenobarbital, the prototype of a highly potent nonsteroidal hepatic microsomal enzyme inducer, is totally ineffective as a prophylactic against this nitrogen mustard. Evidently, there exist great differences in the activity spectrum of various catatoxic substances and much further work will be required to elucidate the biochemical basis of this type of protection.

However, for the clinicians and pathologists, perhaps the most interesting aspect of the work with catatoxic steroids is that it reveals a new mechanism through which "pluricausal diseases" may develop. As we have shown elsewhere (Selye, 1966b) administration of certain sensitizers can establish a predisposition for a particular type of reactivity (e.g., calcification, thrombosis, hemorrhage or necrosis). After such pretreatment, identical morbid lesions can be produced by different "challengers" which merely trigger the eruption of the latent reaction and determine its localization. Thus, in animals given an intravenous injection of lead acetate, calcification will develop at subcutaneous sites treated with many agents, e.g. histamine. If we knew nothing about the pretreatment with lead acetate we would be tempted to consider histamine as a calcifying agent, whereas an antihistaminic (which prevents this reaction) could be regarded as a prophylactic against calcinosis. Actually, here both the production and the prevention of calcification are conditional upon another factor, in this case the presence of excess lead.

We have learned that the possibility of producing hemopoietic tissue damage with hemorrhages and infections by cyclophosphamide, intestinal ulcers by indomethacin, convulsions by digitoxin, anesthesia by barbiturates or calcinosis by DHT depends largely upon the efficiency of hepatic microsomal enzymes. The same is true of the prevention of any among these lesions by catatoxic steroids. It would be false to think of PN as a drug which prevents convulsions, anesthesia, calcification or intestinal ulceration as the single observation of the facts would suggest. We must always remember the many indirect ways in which apparently specific pathogens or therapeutic agents may exert their effects, before concluding that they act upon the target organ which shows the response.

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